The Immunosuppressive Role of IL-32 in Lymphatic Tissue during HIV-1 Infection

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The Immunosuppressive Role of IL-32 in Lymphatic Tissue during HIV-1 Infection

Anthony J. Smith,* Chad M. Toledo,* Stephen W. Wietgrefe,* Lijie Duan,* Timothy W. Schacker,† Cavan S. Reilly,‡ and Ashley T. Haase*

One pathological hallmark of HIV-1 infection is chronic activation of the immune system, driven, in part, by increased expression of proinflammatory cytokines. The host attempts to counterbalance this prolonged immune activation through compensatory mediators of immune suppression. We recently identified a gene encoding the proinflammatory cytokine IL-32 in microarray studies of HIV-1 infection in lymphatic tissue (LT) and show in this study that increased expression of IL-32 in both gut and LT of HIV-1–infected individuals may have a heretofore unappreciated role as a mediator of immune suppression. We show that: 1) IL-32 expression is increased in CD4+ T cells, B cells, macrophages, dendritic cells, and epithelial cells in vivo; 2) IL-32 induces the expression of immunosuppressive molecules IDO and Ig-like transcript 4 in immune cells in vitro; and 3) in vivo, IL-32-associated IDO/Ig-like transcript 4 expression in LT macrophages and gut epithelial cells decreases immune activation but also may impair host defenses, supporting productive viral replication, thereby accounting for the correlation between IL-32 levels and HIV-1 replication in LT. Thus, during HIV-1 infection, we propose that IL-32 moderates chronic immune activation to avert associated immunopathology but at the same time dampens the antiviral immune response and thus paradoxically supports HIV-1 replication and viral persistence. The Journal of Immunology, 2011, 186: 6576–6584.
and that IL-32 expression is associated with a dampening of the antiviral immune response by reducing cell-mediated cytotoxicity, potentially accounting for the correlation between increased IL-32 levels and higher HIV-1 replication in vivo. We thus propose that the nominally proinflammatory cytokine IL-32 actually functions as a double-edged sword during HIV-1 infection, suppressing both immune activation and the antiviral immune response, thereby supporting HIV-1 replication and viral persistence.

Materials and Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of the University of Minnesota. All patients provided written informed consent for the collection of samples and subsequent analysis.

Gut and lymph node biopsy specimens

Ileal, rectal, and inguinal lymph node biopsies from 4 HIV-negative individuals and 26 untreated HIV-1–infected individuals at different clinical stages were obtained for this University of Minnesota Institutional Review Board–approved study. Viral load measurements were obtained the same day as biopsies. Each biopsy was immediately placed in fixative (4% neutral buffered paraformaldehyde or Streck’s tissue fixative) before fixation in 4% neutral buffered paraformaldehyde or Streck’s tissue fixative and mounted using Permount (Fisher Scientific, Pittsburgh, PA), and mounted using Permount (Fisher Scientific, Pittsburgh, PA). Stained sections were examined either by light microscopy or immunofluorescent confocal microscopy at ambient temperatures. Light micrographs were taken using an Olympus BX60 upright microscope (Olympus) with the logarithm of IL-32 expression as the response variable, stage as a four-level factor explanatory variable, and anatomical location as a dichotomous variable to distinguish lymph node from gut tissue (we included additive random effects for subjects to model the within-subject correlation). This model found significant differences between all HIV+ stages and the uninfected subjects (all p < 0.0001) as well as a significant difference between lymph node and gut; however, no significant differences were detected between the HIV+ stages. To test for an effect of day and dose in the in vitro experiments, a two-way ANOVA was conducted using the logarithm of the expression levels. To test for associations between IL-32 and IDO or ILT4, linear models were fit for which the log of IL-32 was the outcome and infection status plus the log of IDO or ILT4 were covariates in addition to the interaction between the two variables. This model found significant positive associations between IL-32 and IDO (p = 0.003), IL-32 and ILT4 (p < 0.0001), and IDO and ILT4 (p < 0.0001) among HIV-1–infected individuals. Associations between continuous variables (IL-32 and immune activation markers, cell proliferation markers, cytotoxic mediators, or CD4+ T cell counts) were estimated using Pearson’s correlation coefficient and tests conducted using a usual t test for a correlation. All calculations were conducted using the statistical software R version 2.10.1.

Microarray data accession number

All microarray results have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE16363).

Results

IL-32 expression is increased in lymph node and gut during HIV-1 infection

To examine changes in IL-32 expression during HIV-1 infection, we used Abs recognizing IL-32 to stain gut (ileum and rectum) and lymph node (inguinal) biopsies from uninfected and HIV-1–infected individuals in each clinical stage of disease: acute (defined as individuals sampled within 4 mo of documented seroconversion), asymptomatic (defined as individuals infected for at
least 4 mo with a CD4+ T cell count >200 cells/μl), and AIDS (defined as infected individuals with a CD4+ T cell count <200 cells/μl) (Table I). Compared with uninfected individuals, levels of IL-32 were significantly increased in both gut and LT during all stages of HIV-1 infection, with the highest IL-32 levels in the acute stage of disease for gut (4.8-fold increase) and AIDS stage of disease for lymph node (5.8-fold increase) (Fig. 1).

To determine the cellular source(s) of IL-32 within gut and LT, we used Abs to CD4 or CD8 (T cells), CD163 (macrophages), CD20 (B cells), CD11c (dendritic cells), killer cell lectin-like receptor subfamily C, member 1 (NK cells), and cytokeratin (epithelial cells) to colocalize IL-32 with the cell types producing this cytokine. As shown in representative images in Fig. 2, IL-32 was expressed during HIV-1 infection in CD4+ T cells, B cells, macrophages, and dendritic cells; minimal expression was detected in CD8+ T cells or NK cells (data not shown). Additionally, IL-32 was highly expressed in the mucosal epithelium of HIV-1-infected gut. Finally, the majority of IL-32+ cells were also expressing IL-18, a proinflammatory cytokine implicated in initiating IL-32 expression (12) (Supplemental Fig. 1). Thus, IL-32 is significantly increased during HIV-1 infection and broadly expressed in many cell types in both gut and LT.

**IL-32 induces IDO expression**

IL-32 has been categorized as a proinflammatory cytokine due to its elevated levels in various inflammatory diseases (13–15) as well as its ability in vitro to induce other proinflammatory mediators, such as TNF-α, IL-1β, and IL-8 (12, 13, 16). Despite IL-32’s association with inflammation and induction of proinflammatory cytokines, recent work has also suggested a role for IL-32 in immune suppression through its ability to stimulate IL-10 production (17). IL-32 also activates immune cells such as the ILT receptors (25).

We then showed that IL-32 expression correlated with IDO production in vivo. We stained for IDO in a subset of study individuals and found a similar pattern of protein expression as for IL-32 (Fig. 4A), resulting in a significant positive association between IL-32+ cells and IDO expression (r = 0.6850, p = 0.003) (Fig. 4B). Moreover, IDO often was coexpressed with IL-32 (Fig. 4C), predominantly in LT macrophages (Fig. 4D) and gut epithelial cells (Fig. 4A). Thus, increased expression of IL-32 during HIV-1 infection coincides with induction of the tryptophan-degrading enzyme IDO in diverse anatomical compartments such as the gut and lymph node, thereby lowering environmental tryptophan (20), which can inhibit immune cell proliferation/activation (21, 22), promote the generation of T Reg cells (23, 24), and stimulate expression of other immunosuppressive molecules such as the ILT receptors (25).

**IL-32 induces ILT4 expression**

ILT receptors are cell-surface inhibitory molecules that bind MHC class I and render target cells anergic (26). Because we had

**Table I. Clinical characteristics of study subjects**

<table>
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<tr>
<th>Patient No.</th>
<th>Disease Stage</th>
<th>Gender</th>
<th>Age (y)</th>
<th>Race</th>
<th>CD4+ T Cell Count (Cells/μl)</th>
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previously identified ILT4 from our microarray analysis as a gene increased in expression in LT during HIV-1 infection (6), we examined the possibility that IL-32–induced IDO might be creating a local environment in LT that favors ILT4 expression. We first tested in vitro whether IL-32 could induce ILT4 expression in PBMCs. As we had shown for IDO induction, IL-32 also increased ILT4 expression in PBMCs (∼2.6-fold; \( p < 0.001 \)) (Fig. 5). In vivo, we also found a similar pattern of protein expression for IL-32 and ILT4 (Fig. 6A), resulting in a significant positive association between IL-32+ cells and ILT4 expression (\( r = 0.9246, p < 0.0001 \)) (Fig. 6B). Moreover, ILT4 often was coexpressed with IL-32 (Fig. 6C), predominantly in LT macrophages (Fig. 6D) and gut epithelial cells (Fig. 6A). Not surprisingly, ILT4 and IDO were also significantly associated with one another (\( r = 0.8790, p < 0.0001 \)) and expressed within the same cell (Supplemental Fig. 3A, 3B). Thus, increased expression of IL-32

FIGURE 1. IL-32 expression is significantly increased in both gut and lymph node during HIV-1 infection. A, Representative immunohistochemical images reveal increased IL-32 expression in the gut and inguinal lymph node during HIV-1 infection (IL-32-positive cells appear brown, whereas cell nuclei appear blue). Original magnification \( \times 400 \). Scale bars, 50 \( \mu m \). B, IL-32 expression was quantified in each biopsy and reported as percent tissue area positive for IL-32. The results are shown with significance where applicable. Symbols: circles, triangles, and squares represent inguinal lymph node, ileal, and rectal biopsies, respectively, whereas the black bars denote the mean expression level of IL-32 in each stage of disease. *** \( p < 0.0001 \). Asympt., asymptomatic.

FIGURE 2. IL-32 is expressed in CD4+ T cells, B cells, macrophages, dendritic cells, and epithelial cells during HIV-1 infection. Representative immunofluorescent images of IL-32 (red staining) and various cell-surface markers (green staining) in the inguinal lymph node and gut from HIV-1–infected individuals, showing colocalization between IL-32 and CD163+ macrophages, CD4+ T cells, CD20+ B cells, CD11c+ dendritic cells, and cytokeratin+ epithelial cells. The insets show the total numbers of cells (cell nuclei appear blue) in each image. Original magnification \( \times 600 \). Scale bars, 10 \( \mu m \).

FIGURE 3. IL-32γ stimulates the production of IDO by both polymorphonuclear cells and PBMCs. A, Representative immunohistochemical images reveal increased IDO expression in polymorphonuclear cells and PBMCs treated with 50 ng IL-32γ (IDO-positive cells appear brown, whereas cell nuclei appear blue). A dose-response curve was initially used (5, 50, and 500 ng IL-32γ) in designing these in vitro experiments. There was no appreciable change in cell markers at 5 ng compared with untreated controls. However, 50 and 500 ng yielded significant changes in IDO expression compared with untreated controls, with little difference between the two dosages. Thus, we report a time-dependent effect of 50 ng IL-32γ on IDO expression. Original magnification \( \times 400 \). Scale bars, 50 \( \mu m \). B, IDO-positive cells were enumerated for each condition and reported as fold-change in IDO production. Data are expressed as the mean ± SEM for which three independent experiments were performed in triplicate. Black bars represent PBMCs, whereas hashed bars represent polymorphonuclear cells. PBMCs were treated with 5 \( \mu g \) PHA as a positive control. The results are shown with significance where applicable. ** \( p < 0.001 \).
data were expressed as the mean ± SEM for which three independent experiments were performed in triplicate. The results are shown with significance where applicable. **<i>p</i> < 0.001.

**FIGURE 4.** Increased IDO expression is significantly associated with IL-32 production in both gut and lymph node during HIV-1 infection. A, Representative immunohistochemical images reveal increased IDO expression in the gut and inguinal lymph node during HIV-1 infection (IDO-positive cells appear brown, whereas cell nuclei appear blue). B, IL-32 expression was significantly correlated with IDO expression in both gut and inguinal lymph node during HIV-1 infection. Symbols: circles and triangles represent inguinal lymph node and ileal biopsies, respectively. C and D, Representative immunofluorescent images of IDO (red staining) and IL-32 or CD163 (green staining) in the inguinal lymph node from HIV-1-infected individuals, showing colocalization between IDO and IL-32 and between IDO and CD163+ macrophages. The insets show the total numbers of cells (cell nuclei appear blue) in each image. Original magnification ×400. Scale bars, 50 μm.
IDO and ILT4. This observation complements a previous study in which IL-32 was shown to induce the immunosuppressive cytokine IL-10 (17).

The balance between immune activation and immune suppression is critical throughout the immune system, whereby perturbation of this balance can have deleterious, immunopathological consequences for the host (30). Additionally, this balance is crucial in determining the effectiveness and ability of the immune system to initially clear acute infections or partially control persistent infections. Because HIV-1 is usually not cleared in the acute stage of infection (31), the immune system, without antiviral therapy, is confronted over a period of years with the sustained challenge of managing this balance between chronic immune activation, needed to maintain host defenses to partially control persistent infections.

**FIGURE 6.** Increased ILT4 expression is significantly associated with IL-32 production in both gut and lymph node during HIV-1 infection. A, Representative immunohistochemical images reveal increased ILT4 expression in the gut and inguinal lymph node during HIV-1 infection (ILT4-positive cells appear brown, whereas cell nuclei appear blue). B, IL-32 expression was significantly correlated with ILT4 expression in both gut and inguinal lymph node during HIV-1 infection. Symbols: circles and triangles represent inguinal lymph node and ileal biopsies, respectively. C and D, Representative immunofluorescent images of ILT4 (green staining) and IL-32 or CD68 (red staining) in the inguinal lymph node from HIV-1–infected individuals, showing co-localization between ILT4 and IL-32 and between ILT4 and CD68+ macrophages. The insets show the total numbers of cells (cell nuclei appear blue) in each image. Original magnification ×400. Scale bars, 50 μm.

**FIGURE 7.** IL-32 expression in the lymph node of HIV-1–infected individuals is associated with reduced cell proliferation, cell activation, and cytotoxic mediators. IL-32 expression within the inguinal lymph node was inversely correlated with mRNA levels of cell proliferation markers Ki-67 and proliferating cell nuclear Ag, immune activation markers CD38 and HLA-DR, and cytotoxic mediators perforin, granzyme, NK cell group 7 sequence (NKG7), and killer-specific secretory protein of 37 kDa (KSP37). mRNA levels are taken from Li et al. (6).
viral replication, and moderating the immunopathological consequences of this chronic immune activation.

The transcriptional profiles of the acute, asymptomatic, and AIDS stages of HIV-1 infection (6) have revealed the complexity of managing chronic immune activation in persistent infection. Initially, the host’s immune system responds by upregulating expression of large numbers of genes that mediate immune activation and innate and adaptive defenses. With the general failure of these defenses in clearing infection, there is an abrupt decrease in expression of most of these genes to levels indistinguishable from HIV-1–uninfected individuals in the asymptomatic stage of infection, which we have interpreted as immunoregulatory mechanisms mounted by the host to strike a balance between moderating chronic immune activation and maintaining host defenses to partially contain infection.

The mediators of this immunoregulatory transition, however, were not immediately obvious in the lists of genes with altered expression, with the exception of IL-32, IDO, and ILT4 (6), and hence the focus on these genes in the work we now report. From the evidence we present, IL-32 certainly could be one of these early immunoregulatory mediators, with increased expression at the right place and right time—in many immune cells during acute HIV-1 infection in both the gut and lymph node as well as in intestinal epithelial cells (Figs. 1, 2). The induction of IL-32 itself is likely due to other proinflammatory cytokines increased during HIV-1 infection (32), particularly IL-18 (Supplemental Fig. 1), which has been shown to be a potent inducer of IL-32 in vitro (12). The antigenicity of HIV-1 itself is unlikely to stimulate IL-32 production, as Nold et al. (33) demonstrated that infection of PBMCs with various strains of HIV-1 actually inhibited production of this cytokine rather than enhancing it.

We had also observed increased expression of ILT4 and IDO in early HIV-1 infection (6) and conjectured that these immunosuppressors were also partly responsible for the immunoregulatory transition. In this study, we show that IL-32, IDO, and ILT4 are coregulated and associated with decreased immune activation, proliferation, and cytotoxic host factors. In vitro, IL-32 induced both IDO and ILT4 expression in PBMCs, whereas in vivo, the levels of IL-32 in LT and gut correlated with both IDO and ILT4 expression levels and were, in turn, inversely correlated with markers of cell proliferation and cytotoxic NK and T cell markers.

The role of IDO in HIV-1 infection has been well documented (20, 34) in suppressing various arms of the immune system by depleting locally available stocks of the essential amino acid tryptophan. A tryptophan-depleted environment has been repeatedly described in HIV-1 infection (35–38) and thought to be responsible for inhibiting essential cellular functions through its potent antiproliferative and immunosuppressive effects (21, 22,

FIGURE 8. IL-32 expression in the lymph node of HIV-1–infected individuals is associated with productive viral replication, lower granzyme expression, and decreased CD4+ T cell viability. A–D, Representative light micrographs reveal a qualitative association between HIV-1 replication (HIV-1 RNA+ cells appear as collections of black silver grains overlying a single cell, whereby each red arrow denotes a single HIV-1 RNA+ cell; cell nuclei appear blue) and IL-32 expression in the inguinal lymph node of HIV-1–infected individuals. Immunofluorescent images reveal decreased granzyme B expression (green) and lower CD4+ T cell numbers (red) with increasing IL-32 expression in the same lymph nodes. Original magnification ×100 (light micrographs) and ×200 (immunofluorescent micrographs). Scale bars, 100 μm.
39). Additionally, high tryptophan catabolism within the environment can promote the local generation of $T_{reg}$ cells (23, 24) and other immunosuppressive molecules (e.g., ILT4) (25), a process that can lead to further IDO induction (34), resulting in a continuous cycle of immunosuppressive amplification.

Like IDO, ILT4 can also serve to further amplify an immunosuppressive environment; ILT4 expression on APCs such as macrophages can inhibit proliferation of immune cells (26, 40), render immune cells anergic and unresponsive to extracellular stimuli (26, 40, 41), and promote the local generation of $T_{reg}$ cells (25, 41, 42). The localization of ILT4 and IDO in vivo, mainly in macrophages, is consistent with these functions.

We think that IL-32, IDO, I LT4, and $T_{reg}$ cells comprise important components of an immunoregulatory axis designed to counter the pathological effects of chronic immune activation in persistent HIV-1 infections and the prime candidates in pathogenic SIV infections. The price the host pays for these moderating effects is impaired host defenses, ranging from smaller numbers of virus-specific CTLs (27, 43) to the decreased expression of cytotoxic effectors (44–46) associated with increased IL-32 expression. In support of this, a recent study showed that splenocytes isolated from mice infected with IL-32–expressing epithelial cells displayed dampened cell-mediated cytotoxicity compared with splenocytes isolated from control mice (47). Although IL-32 has been shown in vitro to have IFN-mediated antiviral activity (33), we think in vivo that IL-32’s immunosuppressive effects contribute, at best, to partial control of untreated HIV-1 infection. In sum, although it remains difficult to quantify the suppressive contributions from IL-32 itself in terms of overall cell-mediated cytotoxicity compromised during HIV-1 infection (48), these data, nevertheless, suggest that IL-32 may be a contributing factor in an immunoregulatory axis that collectively acts as a double-edged sword in lentiviral immunodeficiency infections.

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Disclosures

The authors have no financial conflicts of interest.

References
